HUMIC ACID INVESTIGATIONS: 3. STUDIES ON THE CHEMICAL PROPERTIES OF CERTAIN HUMIC ACID PREPARATIONS

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Most studies of humic acid from the very early days until recent times have been amply reviewed (4, 5, 6, 12, 13, 43, and fn. [2]). Several quite recent reports, however, deserve attention herein. A paper by Bremner and Shaw states that the nitrogen in a soil humic acid was mineralized at a rate intermediate between that of nitrogen in the form of lignin ammonia and that in the form of lignin protein (4, 5, 6). This would seem to suggest that the behavior of the natural humic acid could be explained by a concept incorporating both the lignin-protein and lignin-ammonia theories.

Most recently, Morrison (36) establishes very strong support for the theory of a lignin origin of humus on the evidence of yield of aldehyde by alkaline nitrobenzene oxidation of humus. Some earlier evidence for the presence of lignin-like materials was the production from humic acid of substances like vanillin (20) and 3:5-dinitroguaicol[3] which can also be produced from lignin.

Swaby[4] (42), finding little evidence for protein (Sevag technique and biuret test) or lignin, examined the browning systems (26) postulated by Dawson (13), but he found that long periods and considerable heat energy were required to produce appreciable amounts of melanoidins. The examination of polymers similar to those proposed by Flaig (20) [*see also* Mason (33)] produced this interesting result: amino acids linked in a quinonoid polymer could be released by acid hydrolysis in much the same way as amino acids can be released from proteins. Swaby, therefore, proposes a humic acid structure based on this concept. That others have found phenols and carboxylic acids among the degradation products of humic acids treated with, for example, sodium and liquid ammonia (14, 29), is interesting.

Although Beutelspacher (3) showed by electron microscopy that humic acids are spherical colloids and thus cannot play the role of soil crumb structure stabilizers after the manner of linear polymers, such as natural polysaccharides or the soil conditioner "Krilium", they appear to serve as an appreciable dynamic reservoir of soil nitrogen (8, 9) as well as contributing to the exchange capacity of the soil (39).

EXPERIMENTAL

Paper chromatography of humic acid hydrolyzates

(a) *Sugars.* Humic acid (900 mg.) from peat was hydrolyzed by 2 N H_2SO_4, heating in a sealed tube for 2 hours at 100°. After filtering the hydrolyzate, it was adjusted to pH 4.5 with saturated barium hydroxide, and centrifuged to remove barium sulphate. The liquid and washings were evaporated to dryness, taken up in water (2 ml.) and deionized by passing first through "Zeo-Karb" 215 and then "De-Acidite E." The liquid was now ready for chromatography. Separation was in one dimension, by descending paper technique using Whatman No. 4 paper with *n*-butanol/acetic acid/water (62/12/26) for the development of, and aniline hydrogen phthalate for the detection of, the sugars (37, 38).
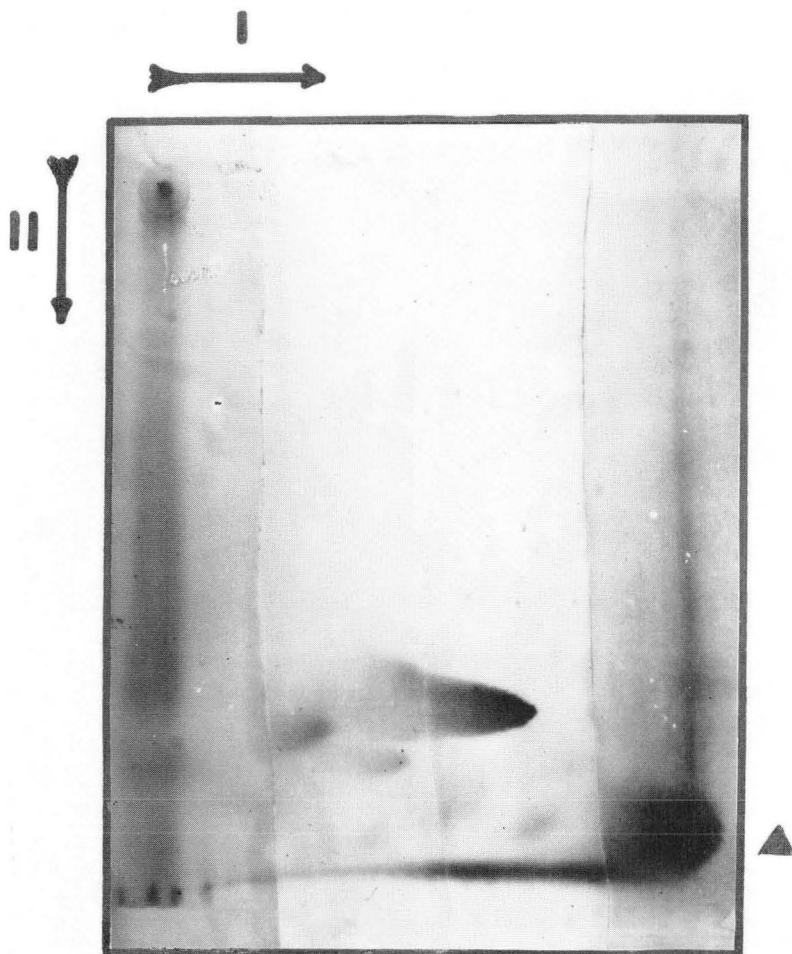
FIG. 1. Two-dimensional chromatogram of phenolic substances present in the electrolytically reduced hydrolyzate of the catechol-glycine model polymer. Detection by the ferric chloride solvents (10): *I*, 2 per cent aqueous acetic acid (3 hrs.); *II*, *n*-butanol/acetic acid/water (4/1/2.2) (10 hrs). The position of the recovered catechol is denoted by *solid triangle*.

(b) *Polyphenols.* The humic acid (100 mg.) from peat was hydrolyzed with 6 N HCl (100°C., 24 hours). The hydrolyzate was filtered and evaporated to dryness *in vacuo*, and the residue dissolved in 10 per cent *iso*-propanol (2 ml.). This was desalted electrolytically, as in the case of amino acids, so reducing quinones to phenols (40, 41). The solution was applied to a Whatman No. 4 paper sheet immediately. A humic acid preparation (50 mg.) from clay soil was treated in a similar fashion.

Two-dimensional descending chromatography was used. The solvents (10) were *n*-butanol/acetic acid/water (40/10/22; 10 hours) and 2

per cent acetic acid (v/v; 3 hours). The polyphenols were revealed by a dip reagent (27) (ferric chloride/potassium ferricyanide).

Examination of a model polymer by methods used for natural humic acids

Preparation of model polymer (10, 17, 18). Catechol (0.2 g.) was dissolved with glycine (0.1 g.) in 0.1 N sodium hydroxide (5 ml. approx.). Potassium persulphate (1 g.), as an oxidant, was added to the solution, which was then carefully heated to boiling. After cooling it was acidified with HCl; the black precipitate

that separated was centrifuged, redissolved, and then reprecipitated (three times). The precipitate was washed three times with dilute HCl, and then thoroughly with ether, prior to hydrolysis (6 N HCl; 100°; 24 hours; sealed tube). The hydrolyzate was evaporated *in vacuo* to dryness, electrolytically desalted (and so reduced) and then examined for amino acids and polyphenols by paper chromatography as before.

RESULTS

Hydrolyzate of humic acids

Analysis for sugars. Only traces of glucose, galactose, arabinose, and xylose were found in the hydrolyzates of humic acid prepared from peat. This is in agreement with the findings of Wright, Schnitzer, and Levick (45).

Analysis for phenolic compounds. Search for phenolic substances in the electrolytically reduced hydrolyzates of humic acids showed: (*a*) In humic acids of peat a single definite phenolic spot (not tyrosine), which occurred in a position near to that of catechol for this solvent; another, less clearly established spot was in one of the chromatograms. (*b*) Humic acid of the clay fraction did not show any single spot. The bulk of the phenolic compounds detected in (*a*) and (*b*) appeared to be present as polymers which ran near the solvent fronts.

Hydrolyzate of a model polymer

Paper chromatographic analysis showed that some of the phenol and the amino acid were recoverable from the hydrolyzate. In addition, other phenolic compounds and ninhydrin reactive substances were detected.

Figure 1 illustrates the chromatogram obtained in examination of the hydrolyzate for catechol and other phenolic compounds. A mixture of compounds appeared as strong spots, and catechol as a very strong spot. Bands at the solvent fronts indicated the occurrence of polymers.

In another chromatogram of the hydrolyzate, glycine and three other ninhydrin reactive spots were detected. A semi-quantitative estimate indicated that at least one-third of the glycine added at the beginning of the oxidation process had been recovered in the hydrolysate of the polymer.

DISCUSSION

Since the amounts of sugar, glucose, galactose, arabinose, and xylose were minute, compared with the amino acids and phenolic substances, sugars do not seem to be an integral part of humic acids, but rather to occur as contaminants absorbed from the fulvic acid fraction. Lynch and co-workers (32) reported 0.55 per cent and 1.96 per cent sugar in humic acids prepared by alkaline extraction of uncultivated and cultivated soils of low organic matter content. These workers precipitated their humic acid preparations only once; comtamination from the fulvic acid fraction would have been greater than in the present studies and would have included microbial polysaccharides (1, 2, 10, 14, 15, 21).

One or two phenolic spots were sometimes detected in humic acid hydrolyzates after electrolytic reduction, but all the reduced hydrolyzates had phenolic polymers present in appreciable amounts.

The release of phenolic polymers by hydrolysis and reduction would appear to be additional evidence for Swaby's[4] (42) theory of the structure of humic acids.

Quinones should be recoverable in monomer and polymer forms, the relative proportions depending on the number which have self-polymerized and the number of quinone nuclei in the self-polymer sections between the amino-acid bridges.

The model experiment with catechol and glycine shows that not only is the amino-acid recoverable by hydrolysis, but the quinone, released by hydrolysis, appears after electrolytic reduction, as the original phenol. Although only roughly one-third of the original amount of glycine and catechol reappeared as such, the rest of the catechol either appeared as other phenolic materials or phenolic polymers, or was oxidized further. Amino acids can be lost through oxidation, as by concentrated hydrogen peroxide (11), and presumably the glycine not accounted for has been oxidized by the potassium persulphate.

Though there appears to be good grounds for Swaby's theory, it may not be the complete answer. Morrison's (36) work would seem to support the lignin origin of humic acids as an alternative. Morrison found that, in humic acid prepared from peats, the amount of aldehydes produced by alkaline nitrobenzene oxidation

was only slightly lower than that from plants growing on the site; but it was considerably lower in humic acids prepared from *mineral* soils, which correspond to the agricultural soils used by Swaby in his investigations. There is evidence, judged from functional group determinations, that the degradation of lignin proceeds further in mineral soils than in peats (22). Although it is not known how far lignin may be modified in the soil before it ceases to yield aldehydes on nitrobenzene oxidation (36), this remains the strongest evidence so far presented of the participation of lignin in humic acid formation.

These two views on humic acid structure need not be mutually exclusive, for in peats we may expect to find organic matter towards the beginning of a process, a process in which the mineral soil organic matter may be considered to be at a much later stage. Since phenolic substances have been produced from peat humic acids by hydrolysis and reduction, there appears to be some part of this humic acid which is built up in the way Swaby suggests. Again, the lignin "linear" polyaromatic structure is known to be degraded eventually by various microorganisms (24), and these work along pathways similar to those degrading condensed aromatic compounds (19) to produce phenolic substances at certain stages.

Evidence is now available[4] to show that leaves contain polyphenols capable of producing tannins and that the site (i.e., general soil reaction) may determine the polyphenol level of the leaf and also, probably, its subsequent fate.

In the past, conflicting claims have been pressed for various theories of humic acid formation without a thorough search being made for some unifying theme in the complexity of facts. Most of the ideas put forward are seemingly based on facts connected with materials of a phenolic nature or capable of acting as a phenolic source. There is no reason why plant and microbe should not synthesize phenolics, or provide materials which can be converted to phenolics by various processes, to give various polymers of a related character.

It has been pointed out (12) that the processes, which have been postulated as producing humic acid, can be separated into three groups, all involving polyphenols or polyphenol-producing materials to a greater or lesser extent. The relative contributions of these processes to humic

acid formation could well depend upon the actual conditions prevailing at the site.

SUMMARY

Swaby claims that amino acids, present as linking molecules between quinonoid structures in humic acid, are recoverable on acid hydrolysis. Evidence is given in this paper in support of his contention, for not only are amino acids released on acid hydrolysis, but, after electrolytic reduction, phenolic substances are found in the hydrolyzates both of natural humic acid (from peats and the clay fraction of a soil) and of a model catechol-glycine oxidation polymer. The presence of sugar molecules in humic acid is examined. The present knowledge of humic acid structure and formation is discussed.

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